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Genomic, phylogenetic and catabolic re-assessment of the *Pseudomonas putida* clade supports the delineation of *Pseudomonas alloputida* sp. nov., *Pseudomonas inefficax* sp. nov., *Pseudomonas persica* sp. nov., and *Pseudomonas shirazica* sp. nov

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ABSTRACT

Bacteria of the *Pseudomonas putida* group are studied for a large panel of properties ranging from plant growth promotion and bioremediation to pathogenicity. To date, most of the classification of individual pseudomonads from this group relies on 16S RNA gene analysis, which is insufficient for accurate taxonomic characterization within bacterial species complexes of the *Pseudomonas putida* group. Here, a collection of 20 of these bacteria, isolated from various soils, was assessed via multi-locus sequence analysis of *rpoD*, *gyrB* and *rrs* genes. The 20 strains clustered in 7 different clades of the *P. putida* group. One strain per cluster was sequenced and results were compared to complete genome sequences of type strains of the *P. putida* group. Phylogenetic analyses, average nucleotide identity data and digital DNA hybridizations, combined to phenotypic characteristics, resulted in the proposition and description of four new species i.e. *Pseudomonas alloputida* Kh7 ^{T} (= LMG 29756 ^{T} = CFBP 8484 ^{T}) sp. nov., *Pseudomonas inefficax* JV551A3 ^{T} (= DSM108619 ^{T} = CFBP 8493 ^{T}) sp. nov., *Pseudomonas persica* RUB6 ^{T} (= LMG 29757 ^{T} = CFBP 8486 ^{T}) sp. nov. and *Pseudomonas shirazica* VM14 ^{T} (= LMG 29953 ^{T} = CFBP 8487 ^{T}) sp. nov.

Introduction

Pseudomonas is one of the most complex and diverse bacterial genera [1,2], encompassing over 250 described species as of May 2018 [3,4] (http://www.bacterio.net/pseudomonas.html). Most species from this genus seem ubiquitous and were isolated from a variety of distinctive habitats in water, soil, and eukaryotic hosts [5]. Although some species or strains were shown to be pathogenic for humans [6], animals [7–9] or plants [10,11], most *Pseudomonas* genotypes are inoffensive as commensal members of the microbiota [2] or even beneficial to their eukaryotic hosts (e.g. plant growth-promoting rhizobacteria [12–14]). Thus, this bacterial taxon presents a large variety of lifestyles, plays diverse roles in biochemical cycles [15,16], and produces various metabolites of biotechnological interest [17], such as vitamin B12 [18], siderophores [19], antibiotics [14,20–22] or phytohormones [20,21,23].

Although species might emerge through ecological specialization [24,25], current bacterial species definition relies on molecular (genomic) homogeneity between strains of the species. In brief, microbial species delineation relies on a polyphasic approach [26,27] based originally on (i) the change in melting temperature (or ΔTm) of heteroduplex DNA formed upon annealing of the DNAs from pairwise-tested strains, and (ii) DNA-DNA hybridization (DDH) percentage [26,27] or whole genome sequence identity computed as average nucleotide identity (ANI) [28]. In addition, sequence comparison of DNA taxonomic markers (house-keeping genes such as *rrs*, *gyrB* or *rpoD*) and characterization of phenotypic traits (morphological, biochemical and/or enzymatic properties) are combined to assemble a set of strains in a species [26,27].

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Over 70 new *Pseudomonas* species have been described in the last ten years [2], and recent analyses based on ANI calculations are suggesting the existence of several uncharacterized species (or genomospecies [29]). Based on multilocus sequence analyses (MLSA) with *rrs*, *gyrB*, *rpoD* and *rpoB* and ANI-based genome comparisons, the *Pseudomonas* genus is divided in three main lineages, each subdivided in several phylogenetic groups : the *P. fluorescens* lineage is constituted of 7 groups, the *P. aeruginosa* lineage 3 groups and the *P. pertucinogena* lineage 1 group [2,30,31].

Within the *P. fluorescens* lineage, the *P. putida* group is the second largest one in the number of described species [32]. These species are mainly studied for their biotechnological potential [33], in relation to the production of particular chemicals [34–36] or phytobeneficial properties [37,38]. *P. putida* was isolated in 1889 [39,40], and since then other species were isolated from clinical samples (*P. mosselii* [41] and *P. monteilii* [42,43]), infected animals (*P. entomophila* L48 [44] and *P. plecoglossicida* [9]), soil (*P. soli* [45], *P. vranovensis* [46], *P. taiwanensis* [47], *P. fulva*, *P. parafulva*, *P. cremoricolorata* [48], and *P. guariconensis* [49]) and water (*P. donghuensis* [50,51]). Many isolates of the *P. putida* group have been classified as *P. putida* strains based on 16S rRNA gene homology. However, most of them do not belong to the *P. putida* species *sensu stricto*, but to genomospecies within the *P. putida* group [51–53]. Recently, Keshavarz-Tohid et al. [54] isolated bacteria from the bean rhizosphere, and 18 of the isolates were affiliated to the *P. putida* group based on phylogenetic analysis of taxonomic markers. These isolates were distributed over five phylogenetic clusters (termed Pp1 to Pp5) that did not include type strains, raising the possibility that they might represent new species within the *P. putida* group.

The objective of this work was to clarify whether the *Pseudomonas* isolates of Keshavarz-Tohid et al. [54] could correspond to new species, and if so to establish these new species in taxonomic terms. To this end, we inferred the phylogeny of the isolates and other strains of the *P. putida* group, sequenced the genome of some of them, and compared *in silico* these bacteria to the type strains from the *P. putida* group and a large panel of strains published as *P. putida* (we will hereafter refer to them as *P. 'putida'* in cases where they were misnamed as *P. putida*) and for which the genomic sequence was available. Thus, the phylogeny of 95 strains of the *P. putida* group was investigated by MLSA, and they were assessed based on average nucleotide identity (ANI), digital DNA hybridizations and phenotyping. This resulted in the proposition of four new species within the *P. putida* group, i.e. *P. alloputida* sp. nov., *P. inefficax* sp. nov., *P. persica* sp. nov. and *P. shirazica* sp. nov.

Material and methods

Bacterial strains, culture conditions and DNA extraction

Bacterial strains were previously isolated from different soils from Iran or France and affiliated to the *Pseudomonas* genus [37,54]. Briefly, 20 strains originated from rhizosphere of bean plants from three Iranian provinces: strains RUB1 (previously VF16) and RUB2 (previously VF13) from Fars province; VKh2, VKh4, VKh9, VKh10, RUB6 (previously VKh13), VKh7, VKh14, VKh17 from Khorasan province ; VM2, VM3, VM10, VM11, VM13, VM14 and RUB5 (previously VM6) from Mazandaran province [54]. Three strains named JV241 A, JV551A1 and JV551A3 were isolated from the rhizosphere of maize grown in French soil (Béligneux, 30 km East from Lyon) [37]. Besides this collection, *P. 'monteilii'* SB3078 [55] (hereafter renamed *P. shirazica*), *P. 'putida'* W15Oct28 [56], *P. 'putida'* BW11M1 [57] (hereafter renamed *P. mosselii*) and *P. cremoricolorata* DSM 17059^T [56] were obtained from Aalborg University (Denmark), Université Libre de Bruxelles (Belgium), Catholic University Leuven (Belgium) and University of Malaya (Malaysia), respectively. The 18 strains were routinely cultivated overnight on King's B [58] or LB [59] media at 28 °C with shaking (150 rpm). Bacterial genomic DNA was extracted from 500 μL of bacterial culture using the NucleoSpin Tissue kit (Macherey-Nagel, Hoerdt, France), following the manufacturer's instructions. DNA was quantified spectrophotometrically and adjusted to 30 ng μL^{-1}.

Phylogenetic analysis

The phylogenetic analyses included 126 *Pseudomonas* strains with complete or draft genome sequences (accession numbers available in Table S1), 13 *Pseudomonas* strains with sequenced taxonomic markers (Table S2; see below), and *Cellvibrio japonicus* Ueda 107^T as outgroup. Nucleotidic sequences were retrieved from Genbank (Table S1) and aligned using MUSCLE v3.8.31 [60]. Alignments were used to compute Maximum Likelihood trees using PhyML [61], with 500 bootstraps, and SeaView v4 [62]. Phylogenetic trees from individual *rrs* (16S rRNA gene), *gyrB* and *rpoB* sequences were generated (not shown), as well as a phylogenetic tree based on concatenated sequences with a total length of 2963 nucleotides (1436 for *rrs*, 820 for *gyrB*, 707 for *rpoD*).

Phenotypic profiling

Strains RUB1, VKh7, RUB6, VKh14, VM14, JV551A1, JV551A3, *P. 'monteilii'* SB3078, *P. 'putida'* W15Oct28, *P. 'putida'* BW11M1 and *P. cremoricolorata* DSM 17059^T were tested for phenotypic characteristics, and results compared with published data on the other strains. Cell morphology and number of flagella were investigated by using the method of Heimbrook et al. [63]. Biochemical tests were done with Biolog GEN III MicroPlate (BIOLOG, Hayward, CA), according to manufacturer's instructions, using 71 carbon sources and 23 chemical sensitivity assays over 48 h. All tests were done two times.

Genome sequencing

As described above, genomic DNA was extracted from strains JV241A, JV551A1, JV551A3, RUB1, RUB6, VM14, VKh7 and VKh14 grown overnight in King's B [58]. The resulting DNA samples were sent to Molecular Research LP (Shallowater, TX, USA), where library preparation was performed using the Nextera DNA sample preparation kit (Illumina Inc., San Diego, CA, USA). Genomic DNA was then sequenced using Illumina MiSeq systems and assembled using SeqMan NGen® version 12.0 (DNASTAR, Madison, WI, USA) with paired-end sequencing parameters on the default settings. Genomeannotation was done with the online MicroScope platform [64]. The draft genome sequences can be found under bioprojects PRJEB24813, PRJEB24814, PRJEB24815, PRJEB25064, PRJEB25066, PRJEB25068, PRJEB25065, PRJEB25067 (Table S1).

Computation of average nucleotide identities and digital DNA-DNA hybridizations

Average Nucleotide Identity (ANI) was calculated for 126 complete or draft genomes with fastANI [65] (Table S3 and Fig. 1). In total, the dataset consisted of 15,750 pairwise values (excluding the 126 pairwise comparisons of each genome with itself). The Genome-to-Genome Distance Calculator GGDC 2.1 [66–68] was used to calculate the digital DNA–DNA hybridization (dDDH) estimates between genomes of candidate strains, so as to define new species in comparison with genomes of type strains from the *P. putida* group.

	Pseudomonas sp. JV241A	***Pseudomonas donghuensis* HYS ᵀ**	***P. vranovensis* DSM 16006 ᵀ**	***Pseudomonas alkylphenolia* KL28 ᵀ**	*Pseudomonas* sp. USDA-ARS-USMARC-56711	***P. cremoricolorata* DSM 17059 ᵀ**	***P. plecoglossicida* NBRC 103162 ᵀ**	***P. taiwanensis* DSM 21245 ᵀ**	*Pseudomonas mosselii* BW11M1	***Pseudomonas mosselii* ATCCBAA 99 ᵀ**	***Pseudomonas soli* LMG27941 ᵀ**	*Pseudomonas soli* RUB1	***Pseudomonas entomophila* L48 ᵀ**	***P. guariconensis* LMG27394 ᵀ**	***Pseudomonas fulva* CIP106765 ᵀ**	***Pseudomonas parafulva* DSM117004 ᵀ**	***Pseudomonas putida* NBRC 14164 ᵀ**	*Pseudomonas* sp. W15Oct28	*Pseudomonas alloputida* VKh14	***Pseudomonas alloputida* VKh7 ᵀ**	*Pseudomonas alloputida* KT2440	***Pseudomonas monteilii* DSM14164 ᵀ**	*Pseudomonas monteilii* SB3078	***Pseudomonas shirazica* VM14 ᵀ**	***Pseudomonas inefficax* JV551A3 ᵀ**	*Pseudomonas inefficax* JV551A1	***Pseudomonas persica* RUB6 ᵀ**
Pseudomonas sp. JV241A	100	93.3	86.9	87.4	82.8	83.2	84.3	83.3	84.7	84.7	83.5	84.5	84.6	81.2	81.9	83.1	84.0	84.1	83.4	83.4	83.5	83.9	84.3	84.2	84.2	84.1	84.5
***Pseudomonas donghuensis* HYS ᵀ**	93.4	100	87.0	87.2	82.8	83.1	84.4	83.4	84.7	84.7	83.6	84.4	84.5	81.0	81.9	83.2	84.0	84.0	83.4	83.4	83.4	83.8	84.3	84.3	84.0	84.1	84.4
***Pseudomonas vranovensis* DSM 16006 ᵀ**	87.0	86.8	100	87.3	82.1	82.4	83.5	82.7	83.7	83.7	82.9	83.5	83.6	79.8	81.5	82.5	83.1	83.4	82.7	82.7	82.8	83.2	83.3	83.4	83.2	83.3	83.5
***Pseudomonas alkylphenolia* KL28 ᵀ**	87.5	87.3	87.3	100	82.2	82.5	83.6	82.8	83.8	83.7	82.6	83.7	83.7	80.2	81.6	82.7	83.2	83.5	82.7	82.8	82.9	83.1	83.4	83.3	83.4	83.3	83.6
Pseudomonas sp. USDA-ARS-USMARC-56711	83.0	82.7	82.1	82.2	100	83.3	83.7	82.8	84.2	84.1	83.2	83.8	84.0	79.6	82.3	83.4	83.3	83.3	82.8	82.8	82.9	83.3	83.6	83.7	83.5	83.5	83.9
***Pseudomonas cremoricolorata* DSM 17059 ᵀ**	83.4	83.2	82.4	82.5	83.3	100	84.4	83.4	84.5	84.5	83.4	84.4	84.3	80.3	82.5	83.8	83.8	83.9	83.5	83.6	83.4	83.7	84.1	84.3	84.0	84.0	84.4
***Pseudomonas plecoglossicida* NBRC 103162 ᵀ**	84.3	84.3	83.5	83.5	83.6	84.1	100	86.5	87.1	87.0	86.2	86.8	86.9	82.1	83.8	84.9	87.3	87.6	86.6	86.6	86.7	87.2	87.7	87.9	87.8	87.8	88.0
***Pseudomonas taiwanensis* DSM 21245 ᵀ**	83.3	83.3	82.8	82.9	83.0	83.2	86.4	100	85.7	85.7	84.9	85.5	85.6	80.8	83.3	84.1	85.8	86.1	85.5	85.5	85.5	86.0	86.5	86.4	86.4	86.4	86.6
Pseudomonas mosselii BW11M1	84.8	84.7	83.7	83.8	84.1	84.5	87.1	85.8	100	99.2	90.7	91.3	89.6	82.6	83.6	85.3	86.2	86.4	85.7	85.7	85.8	86.2	86.8	86.9	86.7	86.7	86.9
***Pseudomonas mosselii* ATCCBAA 99 ᵀ**	84.6	84.7	83.7	83.7	83.9	84.5	87.0	85.7	99.2	100	90.7	91.2	89.6	82.7	83.6	85.3	86.3	86.3	85.8	85.9	86.0	86.8	86.9	86.9	86.9	86.6	87.0
***Pseudomonas soli* LMG27941 ᵀ**	83.7	83.6	82.9	82.9	83.2	83.4	86.2	84.9	90.9	90.8	100	94.7	88.3	70.0	83.0	84.6	85.3	85.5	84.7	84.7	84.8	85.2	85.9	86.2	86.1	86.1	86.3
Pseudomonas soli RUB1	84.4	84.5	83.6	83.7	83.9	84.3	86.9	85.5	91.3	91.3	94.9	100	89.1	82.4	83.4	85.0	86.3	86.3	85.5	85.5	85.7	86.1	86.5	86.7	86.6	86.5	86.8
***Pseudomonas entomophila* L48 ᵀ**	84.4	84.4	83.6	83.6	83.8	84.3	86.8	85.6	89.6	89.5	88.4	89.1	100	82.7	83.4	85.2	86.2	86.3	85.7	85.7	85.8	86.2	86.8	86.8	86.8	86.8	87.0
***Pseudomonas guariconensis* LMG27394 ᵀ**	81.3	80.9	79.9	80.1	79.7	80.0	81.9	81.1	82.9	82.6	70.0	82.4	82.7	100	78.4	80.5	81.5	81.6	81.4	81.4	82.7	81.6	82.3	82.4	82.5	82.4	83.2
***Pseudomonas fulva* CIP106765 ᵀ**	82.0	81.9	81.5	81.5	82.3	82.5	84.0	83.4	83.6	83.5	83.1	83.4	83.4	79.0	100	82.8	84.3	84.4	83.9	84.0	84.0	84.3	84.4	84.5	84.5	84.5	84.6
***Pseudomonas parafulva* DSM117004 ᵀ**	83.3	83.2	82.7	82.9	83.5	83.8	84.9	84.1	85.5	85.4	84.4	85.2	85.4	80.7	82.8	100	84.3	84.4	84.0	84.1	84.0	84.2	84.9	84.8	84.5	84.5	84.8
***Pseudomonas putida* NBRC 14164 ᵀ**	83.9	83.9	83.0	83.2	83.2	83.8	87.2	85.8	86.1	86.3	85.2	86.2	86.1	81.6	84.2	84.3	100	94.7	90.2	90.1	90.2	90.5	90.1	90.2	90.0	89.9	90.2
Pseudomonas sp. W15Oct28	83.9	83.9	83.4	83.5	83.2	83.8	87.4	86.0	86.3	86.2	85.4	86.3	86.3	81.8	84.3	84.3	94.7	100	90.1	90.1	90.1	90.7	90.2	90.4	90.2	90.1	90.6
Pseudomonas alloputida VKh14	83.5	83.4	70.0	82.8	82.6	83.3	86.6	85.5	85.7	85.9	84.4	85.5	85.7	81.5	83.8	83.8	90.2	90.2	100	100	97.1	89.5	89.5	89.6	89.5	89.4	89.7
***Pseudomonas alloputida* VKh7 ᵀ**	83.4	83.4	70.0	82.8	82.6	83.3	86.7	85.5	85.7	85.8	84.6	85.5	85.6	81.2	83.9	83.8	90.3	90.2	100	100	97.1	89.5	89.5	89.7	89.5	89.4	89.8
Pseudomonas alloputida KT2440	83.5	83.5	82.9	83.0	82.9	83.4	86.7	85.5	85.8	86.1	85.0	85.7	85.9	82.7	83.9	84.0	90.3	90.2	97.1	97.1	100	89.7	89.8	89.7	89.6	89.6	89.8
***Pseudomonas monteilii* DSM14164 ᵀ**	83.9	83.9	83.2	83.1	83.2	83.6	87.2	85.9	86.3	86.8	85.2	86.1	86.3	81.3	84.4	84.2	90.6	90.9	89.6	89.6	89.7	100	90.0	90.2	90.0	89.9	90.5
Pseudomonas monteilii SB3078	84.3	84.2	83.3	83.4	83.4	83.9	87.7	86.5	86.9	86.9	86.1	86.6	86.8	82.5	84.3	84.7	90.2	90.3	89.5	89.5	89.8	90.0	100	97.5	94.4	94.4	93.7
***Pseudomonas shirazica* VM14 ᵀ**	84.3	84.2	83.4	83.4	83.5	84.1	87.8	86.4	86.9	86.9	86.1	86.8	86.8	82.1	84.4	84.6	90.2	90.4	89.6	89.6	89.7	90.1	97.5	100	94.7	94.6	93.8
***Pseudomonas inefficax* JV551A3 ᵀ**	84.2	84.0	83.3	83.4	83.5	84.0	87.8	86.3	86.8	87.0	86.0	86.5	86.8	82.1	84.4	84.5	90.0	90.2	89.6	89.5	89.6	90.0	94.3	94.5	100	99.9	93.6
Pseudomonas inefficax JV551A1	84.3	84.1	83.2	83.4	83.4	84.0	87.7	86.3	86.7	86.6	85.9	86.5	86.7	81.9	84.3	84.4	89.9	90.1	89.4	89.4	89.5	89.9	94.3	94.6	99.9	100	93.5
***Pseudomonas persica* RUB6 ᵀ**	84.5	84.5	83.6	83.5	83.7	84.2	87.9	86.5	87.0	87.0	86.1	86.9	87.0	83.0	84.6	84.8	90.4	90.7	89.8	89.7	89.9	90.3	93.8	93.8	93.7	93.7	100

Fig. 1. Genomic relationship between strains in the *P. putida* group based on ANI values (%), which were determined with fastANI [65]. Type strains are in bold and the four new species in red. A more exhaustive comparison is provided in Table S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Results

Phylogenetic classification of strains affiliated to the P. putida group

A phylogenetic tree from concatenated *rrs*, *gyrB* and *rpoD* genes was generated for all 126 strains (Fig. 2). In addition to 17 type strains and our 20 isolates of the *P. putida* group, the tree contains strains from the *P. putida* group for which the sequenced genome is available in a database (some of them shown here to be misnamed as *P. putida*), and a set of representative type strains of the *P. fluorescens* group and the *P. aeruginosa* lineage. The different strains affiliated so far to *P. putida* were retrieved in different *Pseudomonas* clades, with *P. 'putida'* FDAARGOS121 branching within the *P. aeruginosa* lineage, and *P. 'putida'* CBB5 and MC4 5222 within the *P. fluorescens* group.

Only 3 of 65 non-type strains that were confirmed here to belong to the *P. putida* group did correspond to the *P. putida* species (Fig. 2). None of our 20 isolates [37,54] belonged to *P. putida* species; three of them were found close to type strains, i.e. *P. wadenswilersensis* CCOS 864^T [69] for strain JVA241A (96% sequence identity for the concatenated *rrs*, *gyrB* and *rpoD* sequences) and *P. soli* LMG27941^T for cluster-Pp1 strains RUB1 and RUB2 (each with 98% identity). The remaining 17 strains were distributed in four distinct clusters (Pp2, Pp3, Pp4, Pp5) or close to cluster Pp3 (for strain JV551). Although clusters Pp2, Pp3, Pp4 and Pp5 include various sequenced bacteria named *P. putida* or *P. monteilii* so far, none of them contain the *P. putida* type strain (or any other type strain). Based on previous use of multilocus sequence analyses to identify putative new *Pseudomonas* species [53,70], our findings point to the occurrence of four if not five new species in the *P. putida* group, and hereafter we define four proposed species i.e. *P. shirazica* (corresponding to cluster Pp4), *P. alloputida* (for cluster Pp5), *P. persica* (for cluster Pp2) and *P. inefficax* (for French strains of the Lyon area).

Genome sequence-based species delimitation

ANIs calculated using whole genome data for the eight strains sequenced and the 117 sequenced *Pseudomonas* strains retrieved (including 19 type strains) pointed to 33 genomic species (including the 19 species already described), based on ANI values ≥ 95% (Fig. 1 and Table S3). The ANI values strengthened the proposal of the new species *P. shirazica*, *P. alloputida*, *P. persica* and *P. inefficax*, in that strains within each of them displayed ANIs above 95% with one another but that were below the 95% threshold with all other related strains tested (including type strains). This was the case for (i) *P. 'putida'* S16, *P. 'monteilii'* SB3078 and SB3101, and strains VM14, SF1, DLL-E4, S11, HB3267, MO2 and KG4 (now *P. shirazica*), (ii) *P. 'putida'* BIRD-1, S12, PCL1760, YKD221, SJTE-1, DOT-TIE, PDI, TRO1, JCM 9802, B6-2, INSali382, IDAHO, KT2440 and JLR11, and strains VKh7 (CFBP8484) and VKh14 (now *P. alloputida*), (iii) strain RUB6 (now *P. persica*), and (iv) strains JV551A1 and JV551A3 (now *P. inefficax*). Although the ANI value was below the 95% threshold (i.e. 94.9%) when comparing the RUB1 genome (5.5 Mb) with the partially-sequenced genome (only 0.9 Mb released) of *P. soli* LMG 27941^T, the ANI was > 96% when comparing RUB1 to *P. soli* CCOS 191, suggesting that RUB1 is a new strain of the *P. soli* species. The difference in genome sequence availability had probably biased the ANI estimate with the type strain. As the genome sequence of *P. wadenswilerensis* CCOS 864^T (the closest to JV241 A) has not been released, it is not possible to clarify whether JV241 A belongs to *P. wadenswilerensis* or to another, yet-undescribed species.

In addition to ANI comparisons, the dDDH values determined using all type strains (with GGDC 2.1 [66–68]) showed that for strains VKH7 (proposed type strain for *P. alloputida*), VM14 (proposed type strain for *P. shirazica*), JV551A1 (proposed type strain for *P. inefficax*), *P. 'putida'* W15Oct28 and RUB6 (proposed type strain for *P. persica*), the values were all significantly lower than the cut-off of 70% (Table S4). This confirms, together with ANI, that the five

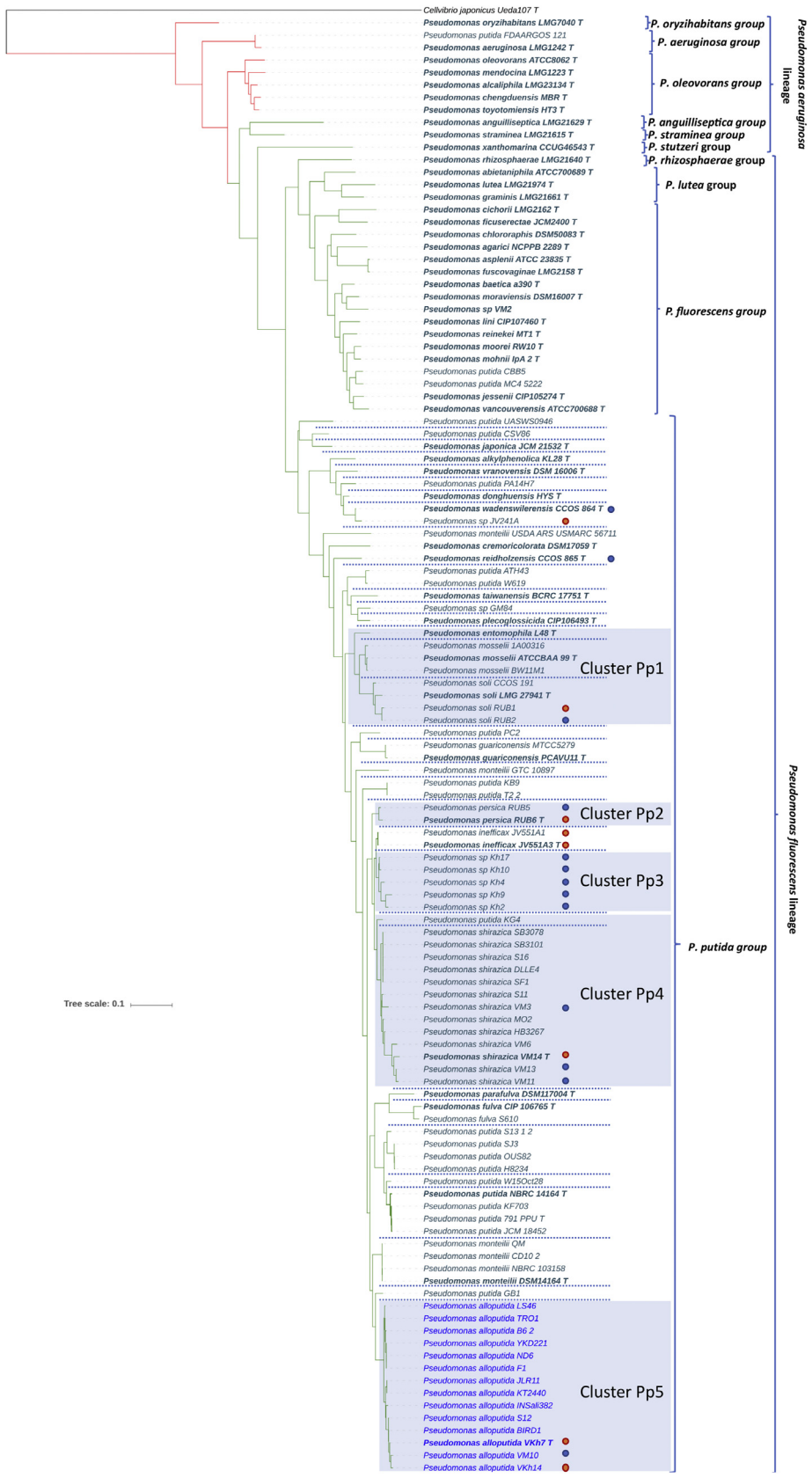

Fig. 2. Maximum Likelihood phylogeny based on concatenated *rrs-gyrB-rpoB* genes of 138 *Pseudomonas* strains, i.e. 126 *Pseudomonas* strains with complete or draft genome sequences (indicated with red dots for genomes obtained during the current project) and 13 *Pseudomonas* strains with sequenced taxonomic markers (blue dots). *Cellvibrio japonicus* Ueda 107^{T} was used as outgroup. In the figure, the groups and phylogenetic lineages proposed previously [2,30,74] are indicated; type strains and proposed type strains are in bold. Within the *Pseudomonas putida* group, the *Pseudomonas putida* clusters proposed previously [54] are highlighted in blue rectangles; the dotted lines are delimiting clusters of strains belonging to the same species according to ANI data. Concatenated sequences were used to compute the tree using PhyML [61], with 500 bootstraps, and SeaView v4 [62]. The tree was visualized using iTOL software [80]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

strains are representatives of novel species in the *P. putida* group. dDDH values calculated with other sequenced strains from the *P. putida* group indicated that two of the proposed new species contain several of these strains, which so far have been incorrectly affiliated to other species (*P. putida* or *P. monteilii* ; see Table S4). It is the case for (i) strains LS46, TRO1, B6-2, YKD221, ND6, F1, JLR11, KT2440, INSali382, DOT-T1E, S12, S12_GCF, BIRD-1, VKh14, IOFA19, PCL1760, PD1, SJTE-1, IOFA1, LF54, H, Idaho and JCM 9802, whose dDDH value with *P. alloputida* VkH7 (type strain) ranges from 70.6% (for TRO1) to ~100% (for VKh14 ; Table S4), and (ii) strains S16, SB3078, SB3101, SF1, DLL-E4, S11, HB3267, MO2 and KG4, whose dDDH value with *P. shirazica* VM14 (type strain) ranges from 78.8% (for S16) to 89.2% (for HB3267).

Morphological and biochemical features

When grown on King's B agar, *P. soli* RUB1, *P. alloputida* VkH7 (proposed type strain), *P. persica* RUB6, VKh14, *P. shirazica* VM14 (proposed type strain), *P. inefficax* JV551A1 (proposed type strain), *P. inefficax* JV551A3, *P. shirazica* SB3078, *P. 'putida'* W15Oct28, *P. mosselii* BW11M1 and *P. cremoricolorata* DSM 17059^T formed circular, convex colonies producing fluorescent pigment(s). All were Gram-negative, aerobic and rod shaped.

Table 1 shows the phenotypic characteristics differentiating the 14 type strains of the *P. putida* group (a more complete set of data is presented in Table S5). In the proposed species *P. alloputida*, strains VKh7 (proposed type strain), KT2440 and VKh14 presented negative results (GEN III MicroPlate, Biolog) for D-melibiose, *p*-hydroxy-phenylacetic acid, α-keto-butyric acid and ß-methyl-D-glucoside tests, whereas its closest relatives *P. putida* and *P. monteilli* were positive for the same tests (Table 1). In the proposed species *P. persica*, strain RUB6 (proposed type strain) gave negative results for bromo-succinic acid, D-galacturonic acid, D-glucuronic acid, D-mannose and *p*-hydroxy-phenylacetic acid tests and positive results for acetic acid, D-serine, glucuronamide and sucrose tests, in contrast to its closest relatives *P. guariconenesis* and *P. inefficax*. In the proposed species *P. inefficax*, strain JV551A3 (proposed type strain) displayed positive results for bromo-succinic acid, D-galacturonic acid, D-glucuronic acid, D-mannose and *p*-hydroxy-phenylacetic acid tests. In addition, *P. inefficax* JV551A1 and JV551A3 differed from *P. alloputida* VKh7 and VKh14 in that their formic acid tests were positive and their sucrose tests negative. In the proposed species *P. shirazica*, strain VM14 (proposed type strain) gave a positive result in the formic acid test and negative results for *N*-acetyl-D-glucosamine, D-galacturonic acid, L-galactonic acid lactone and D-glucuronic acid tests.

For *P. 'putida'* W15Oct28, particular morphological and phenotypic characteristics were found. In contrast to members of related species *P. putida* and *P. fulva*, strain W15Oct28 could not grow at 37 °C and harbored 2 or 3 polar flagella. Contrarily to *P. alloputida* VKh7 and VKh14, strain W15Oct28 gave a positive result for *p*-hydroxy-phenylacetic acid test and negative results for sucrose, D-mannose and D-galacturonic acid tests. Taken together, genomic and phenotypic data are suggesting that strain W15Oct28 is a member of a new genomic species.

Discussion

Members of the *P. putida* group play important ecological roles in various ecosystems, especially in soils and sediments, and have received considerable research attention for plant growth promotion, biodegradation of organic contaminants, and other biotechnological usages [71,72]. The prominent strain KT2440 (i.e. mt-2 = DSM 6125 = ATCC 47054) alone (cluster Pp5; reclassified as *P. alloputida* in this work) has been the focus of several hundred publications.

In 2011 [71], genomes of strains F1, KT2440, W619 and GB-1 were compared as member of a same species, but in the present work we demonstrated that strains F1 and KT2440 are members of the *P. alloputida* species (cluster Pp5), whereas GB-1 (close to cluster Pp5) and W619 (branching between *P. reidholzensis* CCOS 865^T and *P. taiwanensis* BCRC 17751^T) are strains representing two new genomic species. Moreover, GB-1 represents the closest genomic species to *P. alloputida* and strain W619 together with strain ATH43 form another genomospecies more distantly related to *P. putida* since several other species are branching between W619 and *P. putida sensu stricto* (see Fig. 2 and Table S3). Interestingly, Wu et al. [71] noticed that W619 presented the most different genome architecture (synteny around the replication origin is not conserved with the other strains) and heavy metal gene organization compared to *P. alloputida* strain KT2440 and strain F1 or to *P. 'putida'* GB-1. Similarly, Lidbury et al. [73] analyzed the phosphorous scavenging capabilities of different *Pseudomonas* species and grouped strains in different species, with strains KT2440 and BIRD-1 together (now in *P. alloputida*), and GB-1 in a new genomic species and W619 in another new genomic species.

The taxonomy of *P. putida* and related species has long been recognized in need of a clarification [43,51,74], and it is in this context that the current taxonomic assessment was carried out. Comparative genomic analysis using all the genome sequences available in public databases enabled to define 33 genomic species encompassing the 19 already described species (Figs. 1 and 2, Tables S3 and S4). The other 14 genomic species (currently unnamed) encompassed strain with incorrect species affiliations (Table 2). Four genomic species among the 14 were assessed by differential phenotypic analysis, which enabled to propose four new species.

Taxonomic characterization of the new isolates

Phylogenetic and genomic analyses showed that certain isolates could be classified as members of a new *Pseudomonas* species, and strain Kh7 (= LMG 29756 / CFBP 8484; cluster Pp5) was designated as the type strain for *Pseudomonas alloputida* sp. nov. (Table 3). The new species includes strain KT2440, which had been recognized as not belonging to the *P. putida* species [75]. Indeed, DNA–DNA hybridization between strains *P. putida* DSM 291^T and KT2440 was estimated to be 50.5% [75], i.e. below the 70% threshold that delimits bacterial species. The phenotypic characterization shows profiles (Tables 1 and S5) of all representative type strains of the *Pseudomonas putida* group. From the main characteristics, the *P. alloputida* species is characterized by Gram-negative cells forming white colonies on Kings' B medium, approximately 3 mm in diameter after 24 h growth. The cells were rods, motile and 1.6–5 μm in length, with a growth temperature optimum of 28 °C and an optimum pH of 7.0. Their genome size ranged from 5.7 to 6.48 Mb with a GC% between 61.39% and 61.99% (Tables 4, S6 and S7).

Phenotypic and phylogenetic analyses enabled to propose strain RUB6 (= LMG 29757/CFBP 8486; cluster Pp2) as the type strain of the new species *Pseudomonas persica* (detailed characteristics are given in Table 5). From the main characteristics, *P. persica* corresponds to Gram-negative cells forming white colonies on Kings' B medium, approximately 3 mm in diameter after 24 h of growth. Compared with neighboring (sister) species found in the MLSA tree (Fig. 2), *P. persica* cannot use bromo-succinic acid, D-galacturonic acid, D-gluronic acid but was able to use sucrose (unique among the tested species of the *P. putida* group). The cells were rods, motile, and 1.6–5 μm in length, with an optimum growth temperature of 28 °C and optimum pH of 7.0. Genome size was 5.42 Mb, with a GC% of 62.92% (Tables 4, S6 and S7).

Table 1
Selected differential phenotypic characteristics of species of the *P. putida* group. Data are shown for the type strain or (when available) the mean data of type strain and related strains of the same species. Literature data are shown for *P. plecoglossicida* ATCC 700383 ^{T} [9], *P. guariconenesis* PCAVU ^{T} [49], *P. parafulva* DSM 17004 ^{T} [44], *P. fulva* IAM 1529 ^{T} [44], *P. entomphila* L48 ^{T} [44], *P. putida* ATCC 12633 ^{T} [44] and *P. monteilii* ATCC 700476 ^{T} [44]. A full list of tested phenotypes is given in Table S5. Presented data were determined via GEN III MicroPlate (Biolog) metabolic tests. Type strains are in bold.

	P. plecoglossicida ATCC 700383 ^{T}	***P. entomphila*** L48 ^{T}	*P. mosselii*[a]	*P. soli*[b]	***P. guariconenesis*** PCAVU ^{T}	*P. persica*[c]	*P. inefficax*[d]	*P. shirazica*[e]	***P. parafulva*** DSM 17004 ^{T}	***P. fulva*** IAM 1529 ^{T}	*Pseudomonas* sp. W15Oct28	***P. putida*** ATCC 12633 ^{T}	***P. monteilii*** ATCC 700476 ^{T}	*P. alloputida*[f]
Flagellation	multiple polar	single polar	single polar	single polar	two polar	single polar	single polar	single polar	single polar	single polar	2 to 3 polar	single polar	ND	single polar
GEN III MicroPlate (28 °C)														
6% NaCl	–	+	+	+	ND	–	–	–	+	+	–	–	+	–
Acetic acid	+	+	+	+	–	+	+	+	+	+	–	+	+	+
Bromo-succinic acid	+	+	+	+	W	–	+	–	+	+	–	+	+	D
D-Arabitol	–	+	+	–	–	–	–	–	–	–	–	–	–	–
D-Fructose	D	+	+	+	W	+	+	+	–	–	+	+	+	+
D-Galacturonic acid	–	–	–	–	–	–	+	–	–	–	–	+	+	+
D-Glucuronic acid	D	–	–	–	–	–	+	–	–	–	+	+	+	+
D-Mannitol	–	+	+	+	–	–	–	–	–	–	–	–	–	–
D-Mannose	+	+	+	+	–	–	+	+	+	+	–	+	+	+
D-Melibiose	+	–	–	–	–	–	–	–	–	W	–	+	+	–
D-Serine	+	+	–	–	–	+	+	D	+	+	+	+	+	D
D-Sorbitol	+	–	–	–	–	–	–	–	–	–	–	–	–	–
Dextrin	+	–	–	–	–	–	–	–	–	–	–	+	+	–
Glucuronamide	–	–	D	–	–	+	+	+	ND	ND	+	+	ND	+
L-Fucose	–	+	W	–	–	–	–	+	–	–	–	–	–	–
L-Pyroglutamic acid	ND	+	+	+	+	+	+	+	ND	ND	–	–	ND	+
p-Hydroxyphenylacetic acid	D	+	+	+	–	–	+	–	+	+	+	+	+	–
Propionic acid	+	+	+	+	+	+	+	+	+	+	–	+	+	+
Quinic acid	+	–	+	+	+	+	+	+	–	–	+	–	+	+
Sucrose	–	–	–	–	–	+	–	–	–	–	–	–	–	D
Tween 40	–	+	+	+	+	W	D	D	+	+	–	+	+	D
α-Hydroxybutyric acid	+	D	W	D	–	–	–	–	+	+	–	D	+	–
α-Keto-butyric acid	D	D	+	–	–	–	D	–	+	+	–	+	–	–
β-Methyl-D-glucoside	D	D	D	D	–	–	–	–	–	W	–	w	+	–
Formic acid	+	+	+	D	–	+	+	+	+	–	–	+	+	D

–, negative; + positive; ND, not done; W, weak; D, depends on the tested strain or experiment.

[a] For *Pseudomonas mosseli*, data for type strain ATCC 700476^{T} [44] and strain BW11M1 were combined.

[b] For *P. soli*, data for type strain F-279, 208^{T} [45] and strain F16 were combined.

[c] For *P. persica*, data for type strain RUB6^{T} and strain VM16 were combined.

[d] For *P. inefficax*, data for type strain JV551A3^{T} and strain JV551A1 were combined.

[e] For *P. shirazica*, data for type strain VM13^{T}, strainVM14 and strain SB3078 were combined.

[f] For *P. alloputida*, data for type strain VKh7^{T}, strain VKh14 and strain KT2440 were combined.

Table 2
Proposed species affiliation of *P. putida* group strains for which the previous name proved incorrect or that were named in this work.

Strain name	Former species affiliation	New species
P. mosselii species		
1A00316	*Pseudomonas putida*	*Pseudomonas mosselii*
250J	*Pseudomonas* sp.	*Pseudomonas mosselii*
BW11M1	*Pseudomonas putida*	*Pseudomonas mosselii*
P. soli species		
RUB1=VF6	*Pseudomonas soli*	*Pseudomonas soli*
CCOS 191	*Pseudomonas soli*	*Pseudomonas soli*
P. guariconensis species		
MTCC5279	*Pseudomonas putida*	*Pseudomonas guariconensis*
P. persica species		
RUB5 =VM6	*Pseudomonas* sp.	*Pseudomonas persica*
RUB6 ^{T}= VKh13 ^{T} =LMG 29757 ^{T} =CFBP 8486 ^{T}	*Pseudomonas* sp.	*Pseudomonas persica*
P. inefficax species		
JV551A1 = CFBP 8492	*Pseudomonas* sp.	*Pseudomonas inefficax*
JV551A3 ^{T} =DSM 108619^{T} =CFBP 8493^{T}	*Pseudomonas* sp.	*Pseudomonas inefficax*
P. shirazica species		
SB3101	*Pseudomonas monteilii*	*Pseudomonas shirazica*
SB3078	*Pseudomonas monteilii*	*Pseudomonas shirazica*
S16	*Pseudomonas putida*	*Pseudomonas shirazica*
DLL-E4	*Pseudomonas putida*	*Pseudomonas shirazica*
SF1	*Pseudomonas putida*	*Pseudomonas shirazica*
S11	*Pseudomonas putida*	*Pseudomonas shirazica*
MO2	*Pseudomonas monteilii*	*Pseudomonas shirazica*
HB3267	*Pseudomonas putida*	*Pseudomonas shirazica*
VM14 ^{T} =LMG 29953^{T} =CFBP 8487^{T}	*Pseudomonas* sp.	*Pseudomonas shirazica*
HB13667	*Pseudomonas putida*	*Pseudomonas shirazica*
P. fulva species		
S610	*Pseudomonas putida*	*Pseudomonas fulva*
P. monteilii species		
B001	*Pseudomonas putida*	*Pseudomonas monteilii*
HB4184	*Pseudomonas putida*	*Pseudomonas monteilii*
P. alloputida species		
LS46	*Pseudomonas putida*	*Pseudomonas alloputida*
TRO1	*Pseudomonas putida*	*Pseudomonas alloputida*
B6-2	*Pseudomonas putida*	*Pseudomonas alloputida*
YKD221	*Pseudomonas putida*	*Pseudomonas alloputida*
ND6	*Pseudomonas putida*	*Pseudomonas alloputida*
ATCC = DSM 6899 =BCRC 17059 =**F1**	*Pseudomonas putida*	*Pseudomonas alloputida*
JLR11	*Pseudomonas putida*	*Pseudomonas alloputida*
ATCC 47054 = DSM 6125 =NCIMB 11950 =**KT2440**	*Pseudomonas putida*	*Pseudomonas alloputida*
INSali382	*Pseudomonas putida*	*Pseudomonas alloputida*
DOT-T1E	*Pseudomonas putida*	*Pseudomonas alloputida*
S12	*Pseudomonas putida*	*Pseudomonas alloputida*
S12_GCF	*Pseudomonas putida*	*Pseudomonas alloputida*
BIRD-1	*Pseudomonas putida*	*Pseudomonas alloputida*
VKh14 =LMG 29758 = CFBP 8485	*Pseudomonas* sp.	*Pseudomonas alloputida*
VKh7 ^{T} =LMG 29756^{T} =CFBP 8484^{T}	*Pseudomonas* sp.	*Pseudomonas alloputida*
IOFA19	*Pseudomonas monteilii*	*Pseudomonas alloputida*
PCL1760	*Pseudomonas putida*	*Pseudomonas alloputida*
PD1	*Pseudomonas putida*	*Pseudomonas alloputida*
SJTE-1	*Pseudomonas putida*	*Pseudomonas alloputida*
IOFA1	*Pseudomonas putida*	*Pseudomonas alloputida*
LF54	*Pseudomonas putida*	*Pseudomonas alloputida*
H	*Pseudomonas putida*	*Pseudomonas alloputida*
Idaho	*Pseudomonas putida*	*Pseudomonas alloputida*
JCM 9802	*Pseudomonas putida*	*Pseudomonas alloputida*
P. putida genomic species 1		
GB-1	*Pseudomonas putida*	*Pseudomonas* sp.
P. putida genomic species 2		
W15Oct2018	*Pseudomonas putida*	*Pseudomonas* sp.
P. putida genomic species 3		
S13 1 2	*Pseudomonas putida*	*Pseudomonas* sp.
SJ13	*Pseudomonas putida*	*Pseudomonas* sp.
OUS82	*Pseudomonas putida*	*Pseudomonas* sp.
H8234	*Pseudomonas putida*	*Pseudomonas* sp.
JCM 18798	*Pseudomonas putida*	*Pseudomonas* sp.
P. putida genomic species 4		
KG4	*Pseudomonas putida*	*Pseudomonas* sp.
P. putida genomic species 5		
T2 2	*Pseudomonas putida*	*Pseudomonas* sp.
KB9	*Pseudomonas putida*	*Pseudomonas* sp.
P. putida genomic species 6		
GTC 10897	*Pseudomonas monteilii*	*Pseudomonas* sp.
P. putida genomic species 7		
PC2	*Pseudomonas putida*	*Pseudomonas* sp.

Table 2 (*Continued*)

Strain name	Former species affiliation	New species
P. putida genomic species 8		
GM84	*Pseudomonas* sp.	*Pseudomonas* sp.
P. putida genomic species 9		
W619	*Pseudomonas putida*	*Pseudomonas* sp.
SQ1	*Pseudomonas putida*	*Pseudomonas* sp.
ATH43	*Pseudomonas putida*	*Pseudomonas* sp.
P. putida genomic species 10		
USDA-ARS-USMARC-56711	*Pseudomonas monteilii*	*Pseudomonas* sp.
P. putida genomic species 11		
PA14H7	*Pseudomonas putida*	*Pseudomonas* sp.
P. putida genomic species 12		
CBF10-2	*Pseudomonas putida*	*Pseudomonas* sp.
CSV86	*Pseudomonas putida*	*Pseudomonas* sp.
P. putida genomic species 13		
ABAC8	*Pseudomonas putida*	*Pseudomonas* sp.
UASWS0946	*Pseudomonas putida*	*Pseudomonas* sp.
P. putida genomic species 14		
ABAC63	*Pseudomonas putida*	*Pseudomonas* sp.
MR3	*Pseudomonas putida*	*Pseudomonas* sp.

Table 3
Description of *Pseudomonas alloputida* sp. nov. according to Digital Protologue TA00611 assigned by the www.imedea.uib.es/dprotologue website.

Taxonumber	TA00611
Species name	*Pseudomonas alloputida*
Genus name	*Pseudomonas*
Specific epithet	*alloputida*
Species status	sp. nov.
Species etymology	al.lo.pu'ti.da. Gr. masc. adj. *allos*, other; L. fem. adj. *putida*, rotten, stinking; specific epithet of a *Pseudomonas*; N.L. fem. adj. *alloputida*, another *putida*.
Authors	Vahid Keshavarz-Tohid, Jordan Vacheron, Audrey Dubost, Claire Prigent-Combaret, Parissa Taheri, Saeed Tarighi, Seyed Mohsen Taghavi, Yvan Moënne-Loccoz, Daniel Muller
Title	Genomic, phylogenetic and catabolic re-assessment of the *Pseudomonas putida* clade supports the delineation of *Pseudomonas alloputida* sp. nov., *Pseudomonas inefficax* sp. nov., *Pseudomonas persica* sp. nov., and *Pseudomonas shirazica* sp. nov.
Submitter	Daniel MULLER
E-mail of the submitter	daniel.muller@univ-lyon1.fr
Designation of the type strain	VKh7
Strain collection numbers	CFBP 8484 = LMG 29756
16S rRNA gene accession number	LT718459
Genome accession number [EMBL]	PRJEB25065
Genome status	Draft
Genome size	5707279
GC mol %	61.99
Country of origin	Iran
Region of origin	Khorasan Razavi province
Date of isolation	14/07/2014
Source of isolation	Rhizosphere of bean root
Sampling date	12/07/2014
Geographic location	Neyshaboor city
Latitude	36°11′42.5″N
Longitude	58°49′44.8″E
Altitude	1250
Number of strains in study	3
Source of isolation of non-type strains	Bean rhizosphere
Growth medium, incubation conditions	King's B agar (KBA) at 28 °C
Gram stain	Negative
Cell shape	Rod
Motility	Motile
If motile	Flagellar
If flagellated	Single polar
Sporulation (resting cells)	None
Colony morphology	Colonies were fluorescent, approximately 3 mm in diameter, circular and convex with an entire margin
Lowest temperature for growth	15
Highest temperature for growth	30
Temperature optimum	28
pH optimum	7.5
pH category	Neutrophile
Lowest NaCl concentration for growth	1%
Highest NaCl concentration for growth	8%
Relationship to O_2	Halotolerant (optimum <1% NaCl and growth observed at >6% NaCl)
O_2 conditions for strain testing	Aerobe
Positive tests with BIOLOG	Aerobiosis

Table 3 (*Continued*)

Negative tests with BIOLOG	α-Keto-glutaric acid, a-D-glucose, acetic acid, citric acid, D-fructose, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, D-mannose, D-saccharic acid, glucuronamide, glycerol, inosine, L-alanine, L-aspartic acid, L-glutamic acid, L-histidine, L-lactic acid, L-pyroglutamic acid, L-serine, propionic acid, quinic acid, γ-amino butyric acid, β-hydroxybutyric, aztreonam, vancomycin, guanidine HCl, lincomycin, minocycline, niaproof 4, rifamycin SV, tetrazolium blue, tetrazolium violet, troleandomycin, 1% NaCl, 1% sodium lactate, 4% NaCl, 8% NaCl, D-serine, D-fucose, fusidic acid, L-arginine, L-galactonic acid lactone, L-malic acid, lithium chloride, mucic acid, nalidixic acid, pH 5, pH 6, potassium tellurite, sodium bromate, sodium butyrate
Energy metabolism	α-D-lactose, D-arabitol, D-cellobiose, D-galactose, D-glucose-6-PO_4, D-mannitol, D-melibiose, D-raffinose, D-sorbitol, D-trehalose, dextrin, gentiobiose, L-fucose, L-rhamnose, myo-inositol, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *p*-hydroxy-phenylacetic acid, turanose, α-hydroxybutyric acid, α-keto butyric acid, β-methyl-D-glucoside, gelatin, acetoacetic acid, pectin, 3-methyl glucose, D-aspartic acid, D-lactic acid methyl ester, D-malic acid, D-maltose, D-salicin, glycyl-L-proline, *N*-acetyl neuraminic acid, *N*-acetyl-β-D-mannosamine,
Variable tests with BIOLOG	Bromo-succinic acid, D-serine, methyl pyruvate, sucrose tween 40, formic acid
Positive tests with API	ADH, GLU—assim, MNE, GNT, CAP, MLT, CIT, PAC, OX
Negative tests with API	NO_3, TRP, GLU_ Ferm, URE, ESC, GEL, PNPG, ARA, MAN, NAG, MAL, ADI
Energy metabolism	Chemoorganotroph

Table 4
Genome size and GC% content of species in the *P. putida* group. For species that contain more than one sequenced strain, data are shown as means ± standard deviations. Raw data are shown in Table S6.

Species name	Genome size (bp)	GC (%)
Pseudomonas japonica	6.66×10^6	64.16
Pseudomonas alkylphenolia	5.76×10^6	60.63
Pseudomonas vranovensis	5.70×10^6	61.53
Pseudomonas donghuensis	5.64×10^6	62.42
Pseudomonas cremoricolorata	4.66×10^6	63.50
Pseudomonas taiwanensis	5.42×10^6	61.87
Pseudomonas plecoglossicida	5.34×10^6	62.99
Pseudomonas entomophila	5.89×10^6	64.16
Pseudomonas mosselii	$5.81 (\pm 0.33) \times 10^6$	64.35 ± 0.25
Pseudomonas soli[a]	$5.79 (\pm 0.22) \times 10^6$	64.18 ± 0.01
Pseudomonas guariconensis[a]	5.20×10^6	62.48
Pseudomonas persica	5.42×10^6	62.92
Pseudomonas inefficax	$6.06 (\pm 0.18) \times 10^6$	62.80 ± 0.05
Pseudomonas shirazica	$5.99 (\pm 0.25) \times 10^6$	62.45 ± 0.22
Pseudomonas parafulva	5.09×10^6	63.46
Pseudomonas fulva	$4.68 (\pm 0.08) \times 10^6$	61.89 ± 0.12
Pseudomonas putida	$6.27 (\pm 0.10) \times 10^6$	62.12 ± 0.14
Pseudomonas monteilii	$6.19 (\pm 0.25) \times 10^6$	61.57 ± 0.15
Pseudomonas alloputida	$6.06 (\pm 0.27) \times 10^6$	61.70 ± 0.19

[a] Strains for which the entire genome was not sequenced were not included (including the type strains of *P. soli* and *P. guariconensis*).

Table 5
Description of *Pseudomonas persica* sp. nov. according to Digital Protologue TA00711 assigned by the www.imedea.uib.es/dprotologue website.

Taxonumber	TA00711
Species name	*Pseudomonas persica*
Genus name	*Pseudomonas*
Specific epithet	*Persica*
Species status	sp. nov.
Species etymology	per'si.ca. L. fem. adj. *persica*, Persian.
Authors	Vahid Keshavarz-Tohid, Jordan Vacheron, Audrey Dubost, Claire Prigent-Combaret, Parissa Taheri, Saeed Tarighi, Seyed Mohsen Taghavi, Yvan Moënne-Loccoz, Daniel Muller
Title	Genomic, phylogenetic and catabolic re-assessment of the *Pseudomonas putida* clade supports the delineation of *Pseudomonas alloputida* sp. nov., *Pseudomonas inefficax* sp. nov., *Pseudomonas persica* sp. nov., and *Pseudomonas shirazica* sp. nov.
Submitter	Daniel MULLER
E-mail of the submitter	daniel.muller@univ-lyon1.fr
Designation of the type strain	RUB6
Strain collection numbers	CFBP 8486 = LMG 29757
16S rRNA gene accession number	LT718462
Genome accession number [EMBL]	PRJEB25066
Genome status	Draft
Genome size	5425242
GC mol %	62.92
Country of origin	Iran
Region of origin	Khorasan Razavi province
Date of isolation	14/07/2014
Source of isolation	Rhizosphere of bean
Sampling date	12/07/2014
Geographic location	Neyshaboor city

Table 5 (*Continued*)

Latitude	36°11′42.5″N
Longitude	58°49′44.8″E
Altitude	1250
Number of strains in study	2
Source of isolation of non-type strains	Bean rhizosphere
Growth medium, incubation conditions	King's B agar (KBA) at 28 °C
Gram stain	Negative
Cell shape	Rod
Motility	Motile
If motile	Flagellar
If flagellated	Single polar
Sporulation (resting cells)	None
Colony morphology	Colonies were fluorescent, approximately 3 mm in diameter, circular and convex with an entire margin
Lowest temperature for growth	15
Highest temperature for growth	37
Temperature optimum	28
pH optimum	7.5
pH category	Neutrophile
Lowest NaCl concentration for growth	1%
Highest NaCl concentration for growth	4%
Relationship to O_2	Aerobe
O_2 conditions for strain testing	Aerobiosis
Positive tests with BIOLOG	D-Fructose, D-gluconic acid, D-saccharic acid, D-serine, glucuronamide, glycerol, inosine, L-alanine, L-aspartic acid, L-glutamic acid, L-histidine, L-pyroglutamic acid, L-serine, methyl pyruvate, propionic acid, quinic acid, sucrose, γ-amino butyric acid, β-hydroxybutyric, formic acid, vancomycin, guanidine HCl, lincomycin, minocycline, niaproof 4, rifamycin SV, tween 40, aztreonam, acetoacetic acid, tetrazolium blue, tetrazolium violet, troleandomycin,1% NaCl, 4% NaCl, 1% sodium lactate, D-serine, D-fucose, fusidic acid, L-arginine, L-malic acid, lithium chloride, mucic acid, nalidixic acid, pH 5, pH 6
Negative tests with BIOLOG	Bromo-succinic acid, D-arabitol, D-cellobiose, D-galacturonic acid, D-glucose-6-PO_4, D-glucuronic acid, D-mannitol, D-mannose, D-melibiose, D-raffinose, D-sorbitol, D-trehalose, dextrin, gentiobiose, L-fucose, L-rhamnose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, p-hydroxy-phenylacetic acid, turanose, α-hydroxybutyric acid, α-keto butyric acid, β-methyl-D-glucoside, gelatin, pectin, 3-methyl glucose, 8% NaCl, D-aspartic acid, D-lactic acid methyl ester, D-malic acid, D-maltose, D-salicin, glycyl-L-proline, L-galactonic acid lactone, *N*-acetyl neuraminic acid, *N*-acetyl-ß-D-mannosamine, sodium bromate, sodium butyrate, stachyose, D-fructose-6-PO_4
Energy metabolism	Chemoorganotroph

Table 6
Description of *Pseudomonas shirazica* sp. nov. according to Digital Protologue TA00712 assigned by the www.imedea.uib.es/dprotologue website.

Taxonumber	TA00712
Species name	*Pseudomonas shirazica*
Genus name	*Pseudomonas*
Specific epithet	*Shirazica*
Species status	sp. nov.
Species etymology	shi.ra'zi.ca. N.L. fem. adj. *shirazica* pertaining to Shiraz (a city in Iran)
Authors	Vahid Keshavarz-Tohid, Jordan Vacheron, Audrey Dubost, Claire Prigent-Combaret, Parissa Taheri, Saeed Tarighi, Seyed Mohsen Taghavi, Yvan Moënne-Loccoz, Daniel Muller
Title	Genomic, phylogenetic and catabolic re-assessment of the *Pseudomonas putida* clade supports the delineation of *Pseudomonas alloputida* sp. nov., *Pseudomonas inefficax* sp. nov., *Pseudomonas persica* sp. nov., and *Pseudomonas shirazica* sp. nov.
Submitter	Daniel MULLER
E-mail of the submitter	daniel.muller@univ-lyon1.fr
Designation of the type strain	VM14
Strain collection numbers	CFBP 8487 = LMG 29953
16S rRNA gene accession number	LT718474
Genome accession number [EMBL]	PRJEB25068
Genome status	Draft
Genome size	5,514,185
GC mol %	62.84
Country of origin	Iran
Region of origin	Mazandaran province
Date of isolation	15/07/2014
Source of isolation	Rhizosphere of bean
Sampling date	12/07/2014
Geographic location	Behshahr city
Latitude	36°44′54.7″N
Longitude	53°32′42.9″E
Altitude	−15
Number of strains in study	5
Source of isolation of non-type strains	Bean rhizosphere
Growth medium, incubation conditions	King's B agar (KBA) at 28 °C
Gram stain	Negative
Cell shape	Rod
Motility	Motile
If motile	Flagellar
If flagellated	Single polar

Table 6 (*Continued*)

Sporulation (resting cells)	None
Colony morphology	Colonies were fluorescent, approximately 3 mm in diameter, circular and convex with an entire margin
Lowest temperature for growth	4
Highest temperature for growth	37
Temperature optimum	28
pH optimum	7
pH category	Neutrophile
Lowest NaCl concentration for growth	1%
Highest NaCl concentration for growth	4%
relationship to O_2	Aerobe
O_2 conditions for strain testing	Aerobiosis
Positive tests with BIOLOG	Acetic acid, D-fructose, D-gluconic acid, D-mannose, D-saccharic acid, glucuronamide, glycerol, L-alanine, L-aspartic acid, L-fucose, L-glutamic acid, L-histidine, L-pyroglutamic acid, L-serine, methyl pyruvate, propionic acid, quinic acid, γ-amino butyric acid, β-hydroxybutyric, formic acid, vancomycin, guanidine HCl, lincomycin, minocycline, niaproof 4, potassium tellurite, rifamycin SV, tetrazolium blue, tetrazolium violet, troleandomycin, 1% NaCl, 1% sodium lactate, 8% NaCl, D-fucose, L-arginine, L-malic acid, mucic acid, pH 5, pH 6
Negative tests with BIOLOG	bromo-succinic acid, D-arabitol, D-cellobiose, D-galacturonic acid, D-glucose-6-PO_4, D-glucuronic acid, D-mannitol, D-melibiose, D-raffinose, D-sorbitol, D-trehalose, dextrin, gentiobiose, L-rhamnose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *p*-hydroxy-phenylacetic acid, sucrose, turanose, α-hydroxybutyric acid, α-keto butyric acid, β-methyl-D-glucoside, gelatin, aztreonam, acetoacetic acid, pectin, 3-methyl glucose, D-serine, D-aspartic acid, D-lactic acid methyl ester, D-malic acid, D-maltose, D-salicin, glycyl-L-proline, L-galactonic acid lactone, *N*-acetyl neuraminic acid, *N*-acetyl-ß-D-mannosamine, sodium bromate, stachyose, D-fructose-6-PO_4
Variable tests with BIOLOG	D-serine, inosine, tween 40, 4% NaCl, fusidic acid nalidixic acid, sodium butyrate
Energy metabolism	Chemoorganotroph

Comparative genomic analysis enabled to propose *Pseudomonas shirazica* sp. nov, with VM14 (=LMG 29953=CFBP 8487; within cluster Pp4) proposed as the type strain (Table 6). *P. shirazica* encompasses strains that were isolated from various environments and that exhibit a variety of phenotypes. Indeed, strain VM14 was isolated from bean rhizosphere [54], SB3078 and SB3101 degrade benzene, toluene, and ethylbenzene [55,76], S16 degrades nicotine [77], and HB3267 is a clinical isolate with high pathogenic potential [78]. *P. shirazica* is characterized by Gram-negative cells forming white colonies on Kings' B medium, approximately 3 mm in diameter after 24 h of growth. The cells are rods, motile, 1.6–5 μm in length, with a growth temperature optimum of 28 °C and an optimum pH of 7.0. Genome size ranges from 5.51 to 6.48 Mb with a GC% between 61.96% and 63.02% (Tables 4 and S7).

The last newly described species encompasses two strains (JV551A3 and JV551A1; between clusters Pp2 and Pp3; see Fig. 2) isolated from maize rhizosphere [37,79], with strain JV551A3 (= DSM 108619 / CFBP 8493) proposed as type strain for *P. inefficax* (Table 7). None of the two strains presented any plant growth promotion effects when tested on maize or *Arabidopsis* plants [37,79]. Detailed species characteristics are summarized in Table 7. The cells are rods, motile, 1.6–5 μm in length, with a growth temper-

Table 7
Description of *Pseudomonas inefficax* sp. nov. according to Digital Protologue TA00715 assigned by the www.imedea.uib.es/dprotologue website.

Taxonumber	TA00715
Species name	*Pseudomonas inefficax*
Genus name	*Pseudomonas*
Specific epithet	*inefficax*
Species status	sp. nov.
Species etymology	From in.ef'fi.cax. L. fem. adj. *inefficax*, inefficient. The strains have no effect on plant growth
Authors	Vahid Keshavarz-Tohid, Jordan Vacheron, Audrey Dubost, Claire Prigent-Combaret, Parissa Taheri, Saeed Tarighi, Seyed Mohsen Taghavi, Yvan Moënne-Loccoz, Daniel Muller
Title	Genomic, phylogenetic and catabolic re-assessment of the *Pseudomonas putida* clade supports the delineation of *Pseudomonas alloputida* sp. nov., *Pseudomonas inefficax* sp. nov., *Pseudomonas persica* sp. nov., and *Pseudomonas shirazica* sp. nov.
Submitter	Daniel MULLER
E-mail of the submitter	daniel.muller@univ-lyon1.fr
Designation of the type strain	JV551A3
Strain collection numbers	CFBP8493 = DSM 108619
16S rRNA gene accession number	PRJEB24815
Genome accession number [embl]	PRJEB24815
Genome status	Draft
Genome size	6240036
GC mol %	62.85
Country of origin	France
Region of origin	Béligneux "AIN 01"
Date of isolation	14/01/2014
Date of isolation unknown (< yyyy)	2014
Source of isolation	Soil
Sampling date	12/07/2014
Geographic location	Béligneux "AIN 01"
Latitude	45°52'18.9Ñ
Longitude	5°07'18.2Ë
Altitude	Ã270
Number of strains in study	2
Source of isolation of non-type strains	Maize rhizosphere

Table 7 (*Continued*)

Growth medium, incubation conditions	King's B agar (KBA) at 28 °C
Gram stain	Negative
Cell shape	Rod
Motility	Motile
If motile	Flagellar
If flagellated	Single polar
Sporulation (resting cells)	None
Colony morphology	Colonies were fluorescent, approximately 3 mm in diameter, circular and convex with an entire margin
Lowest temperature for growth	4
Highest temperature for growth	37
Temperature optimum	28
pH optimum	7
pH category	Neutrophile
Lowest NaCl concentration for growth	1%
Highest NaCl concentration for growth	4%
relationship to O_2	Aerobe
O_2 conditions for strain testing	Aerobiosis
Positive tests with BIOLOG	D-Arabitol, D-cellobiose, D-glucose-6-PO_4, D-mannitol, D-melibiose, D-affinose, D-sorbitol, D-trehalose, dextrin, gentiobiose, L-fucose, L-rhamnose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, sucrose, turanose, α-hydroxybutyric acid, β-methyl-D-glucoside, gelatin, acetoacetic acid, pectin, 3-methyl glucose, D-aspartic acid, D-lactic acid methyl ester, D-malic acid, D-maltose, D-salicin, glycyl-L-proline, *N*-acetyl neuraminic acid, *N*-acetyl-ß-D-mannosamine, stachyose, D-fructose-6-PO_4
Negative tests with BIOLOG	acetic acid, bromo-succinic acid, D-fructose, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, D-mannose, D-saccharic acid, D-serine, glucuronamide, glycerol, inosine, L-alanine, L-aspartic acid, L-glutamic acid, L-histidine, L-pyroglutamic acid, L-serine, methyl pyruvate, *p*-hydroxy-phenylacetic acid, propionic acid, quinic acid, γ-amino butyric acid, β-hydroxybutyric, formic acid, aztreonam, vancomycin, guanidine HCl, lincomycin, minocycline, niaproof 4, rifamycin SV, tetrazolium blue, tetrazolium violet, troleandomycin, 1% NaCl, 1% sodium lactate, 4% NaCl, D-serine, D-fucose, fusidic acid, L-arginine, L-galactonic acid lactone, L-malic acid, lithium chloride, mucic acid, nalidixic acid, pH 5, pH 6, sodium bromate, sodium butyrate
Variable tests with BIOLOG	Tween 40, α-keto butyric acid
Energy metabolism	Chemoorganotroph

ature optimum of 28 °C and an optimum pH of 7.0. Genome size ranges from 6.42 to 5.88 Mb with a GC% between 62.85% and 62.75% (Tables 4, S6 and S7).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.syapm.2019.04.004.

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